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REGULATORY T CELLS AND HOST ANTI-CML RESPONSES

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14. ABSTRACT
CD4+CD25+FoxP-3+ regulatory T-cells (Tregs) suppress immune responses to "self" antigens, but also have been shown to suppress host anti-tumor responses in several human malignancies, including breast, gastrointestinal, and ovarian cancer. Identification of CML Tregs as suppressors of host anti-CML responses could have a significant impact upon CML treatment strategies. Methods are currently available to selectively suppress T_{regs} and subsequently boost host anti-CML responses. We have examined the CD4+CD25+FoxP-3+ regulatory T-cell population in the peripheral blood from healthy individuals and those with CML using flow cytometry. Our preliminary studies suggest that the Treg population is higher in those with CML (mean percentage of 5.23 vs 6.94). Furthermore, a subject with poorly controlled CML had the highest percentage of circulating Tregs (9.34) suggesting that these cells might be influencing anti-CML host responses. We are examining functional correlates of the Treg population.

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INTRODUCTION.

Although Imatinib Mesylate (Gleevec®) therapy has clearly made a major impact upon the treatment of chronic myelogenous leukemia (CML), it does not eliminate nonproliferating CML stem cells, and, thus, is unlikely to be curative. Currently, because of age, underlying medical conditions and/or lack of donor availability, only a minority of individuals are eligible for curative treatment with allogeneic hematopoietic stem cell transplantation (HSCT). Therefore strategies that increase the potential for disease control or cure for the majority of individuals with CML are sorely needed. CD4+CD25+FoxP-3+ regulatory T-cells (T_{regs}) suppress immune responses to "self" antigens, but also have been shown to suppress host anti-tumor responses in several human malignancies, including breast, gastrointestinal, and ovarian cancer. Identification of CML T_{regs} as suppressors of host anti-CML responses could have a significant impact upon CML treatment strategies. Methods are currently available to selectively suppress T_{regs} and subsequently boost host anti-CML responses. Furthermore, reduction of host suppressor CML T_{regs} could act synergistically with administration of CML targeted vaccines to boost host anti-tumor responses. Such immune strategies could aid in the elimination of residual leukemic stem cells that persist after other treatments.

BODY.

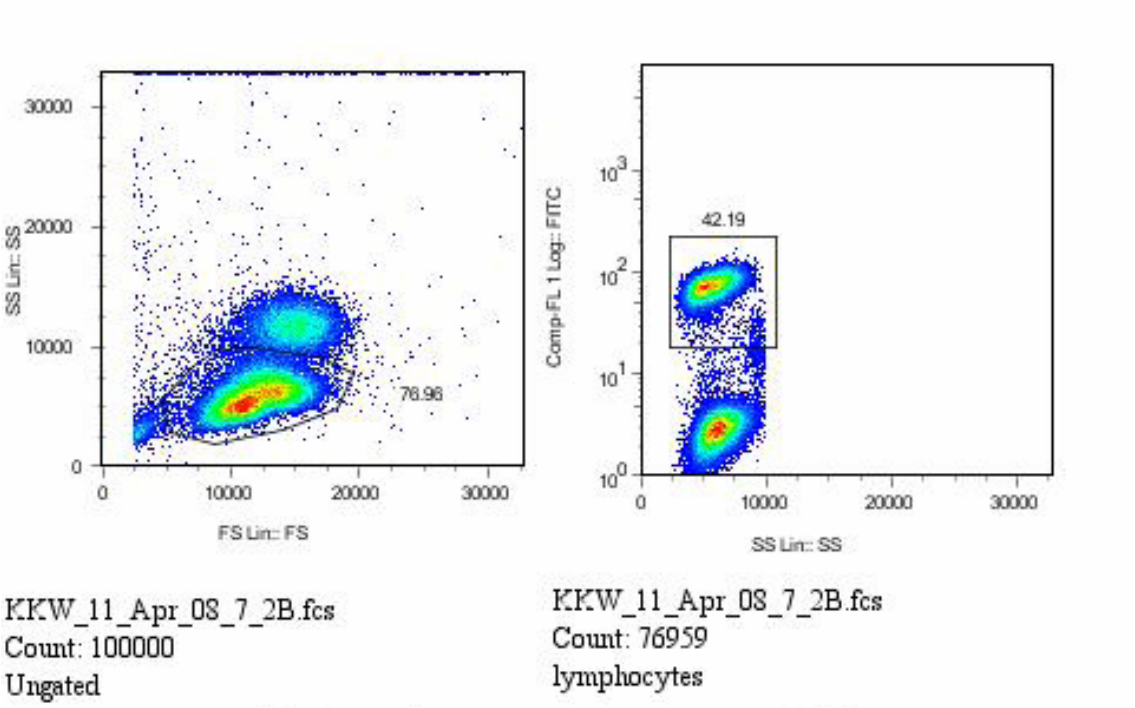
Task 1. **Phenotypic Characterization of CD4+CD25+ T_{regs} in Individuals with Chronic Phase CML:(a) quantification of T_{reg} populations in CML and healthy controls and (b) expression analyses for bcr-abl and FoxP3.** (Months 01-18)

(a) Isolate CD4+ CD25+ T_{regs} from subjects with CML and healthy donor pairs
(b) Test for bcr-abl by PCR and/or fluorescence-in-situ-hybridization (FISH)
(c) Test for FoxP3 by PCR

To assess the potential role(s) of CD4+CD25+FoxP-3+ regulatory T-cells (T_{regs}) in CML, we first examined the T_{regs} peripheral blood profile of healthy individuals compared to those with CML using a City of Hope Institutional Review Board (IRB) approved protocol. Peripheral blood mononuclear cells were isolated from healthy individuals and those with CML, stained with fluorochrome conjugated antibodies against CD4 (FITC), CD25 (PE), and FoxP3 (APC) antigens, characteristic of the regulatory T-cell population, and analyzed using a CyAn™ ADP 9 Color flow cytometer (Beckman Coulter). Conjugated antibodies were obtained from EBiosciences (San Diego, CA), and staining was performed per manufacturer's recommendations.

Cells are first gated using forward and side scatter parameters, with gating of small cells with low granularity consistent with lymphocytes. Compensation was performed to correct for wavelength overlap of the different fluorochromes. Cells were then gated against CD4-FITC, and CD4+ cells were analyzed against CD25-PE and FoxP3-APC to obtain the CD4, CD25, and FoxP3 triple positive population. We initially compared a standard quadrant compared to a freehand (hand drawn) gate for analysis of the CD4+CD25+FoxP-3+ population, and found the latter to be slightly more consistent. Therefore, freehand gates for CD4+CD25+FoxP-3+ cells are depicted in the Figures, and summarized in Table 1, below. It should be noted that all CML subjects that were analyzed to date were on Nolotinib therapy.

Representative examples of profiles from a healthy individual (**Figure 1**) as well as an individual with CML (**Figure 2**) are provided below and overall results are summarized in **Table 1,** below. It should be noted that the individual with CML depicted in Figure 2 was felt to have poorly controlled disease, with 35.8% cells in the periphery which were positive for the BCR-ABL translocation by cytogenetics (FISH).



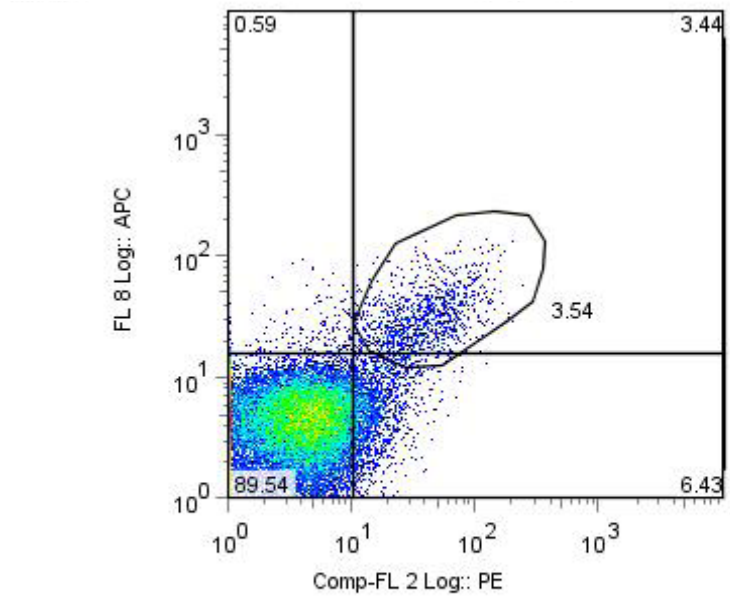
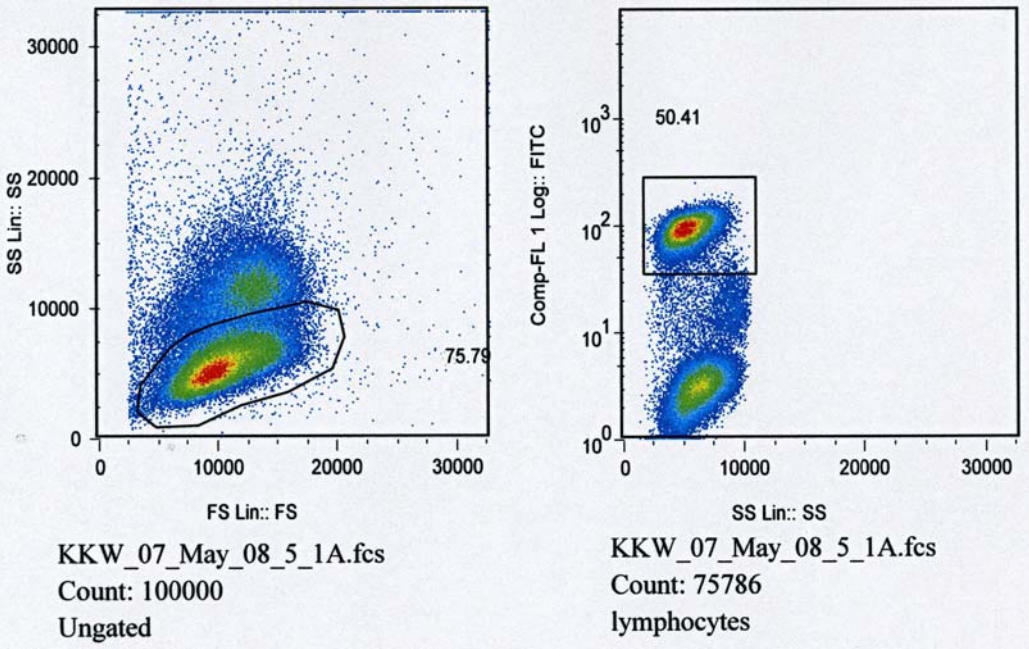


Figure 1. Flow Analysis of CD4+CD25+FoxP-3+ regulatory T-cells (T_{regs}) from a healthy individual. Panel Upper Left. Gate of small, low granularity cells consistent with small lymphocytes. Panel Upper Right. The previously gated cells were analyzed for CD4-FITC expression, and gated (square). Lower Panel. The CD4+ cells were analyzed for CD25-PE and FoxP3-APC expression using a freehand gate; **3.54 %** of CD4+ lymphocytes were CD25 and FoxP-3 positive.



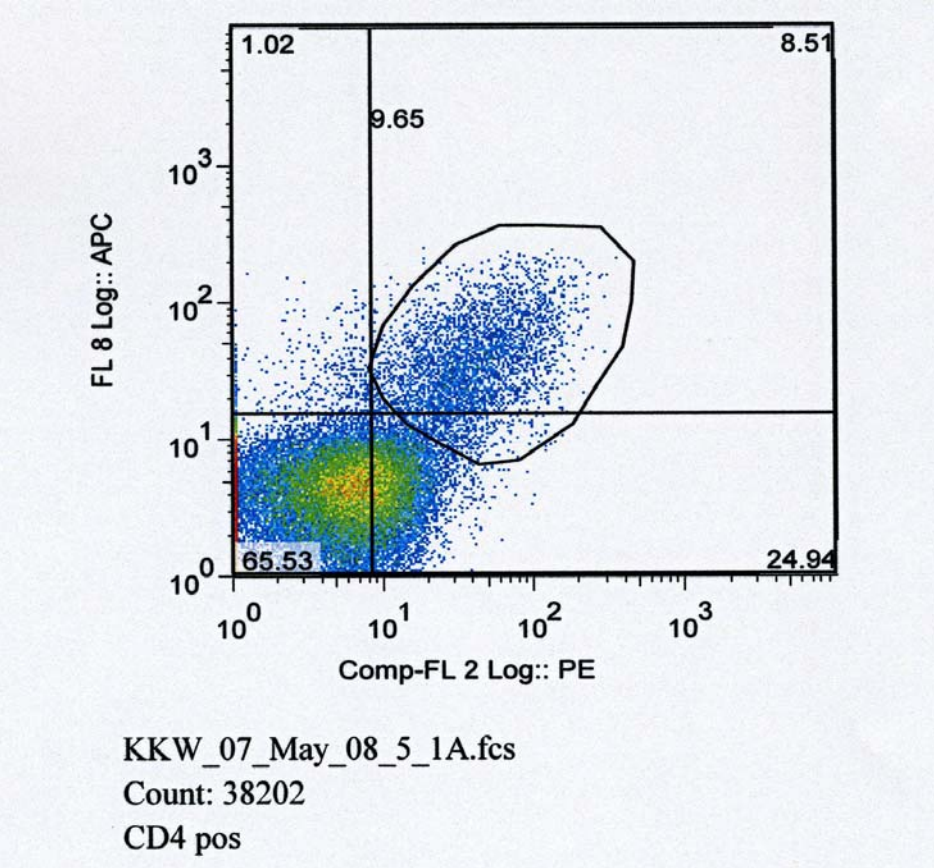


Figure 2. Flow Analysis of CD4+CD25+FoxP-3+ regulatory T-cells (T_{regs}) from an individual with active CML. Panel Upper Left. Gate of small, low granularity cells consistent with small lymphocytes. Panel Upper Right. The previously gated cells were analyzed for CD4-

FITC expression, and gated (square). Lower Panel. The CD4+ cells were analyzed for CD25-PE and FoxP3-APC expression using a freehand gate; **9.65 %** of CD4+ lymphocytes were CD25 and FoxP-3 positive.

TABLE 1

Sample	Gender	Age	Percent CD4+CD25+FoxP3+ Using Freehand Gate	Average	Absolute # of CD4+ Cells	Absolute # of CD25+ & FoxP3+ Cells	Comments
NML-022208-1			5.56		28,961	1,610	
			4.98	5.27	28,508	1,420	
NML-022208-2			3.46		32,890	1,138	
			3.61	3.54	30,966	1,118	
NML-032808-1	F	55	5.63		38,153	2,148	
			5.81	5.72	39,510	2,296	
NML-032808-2	F	35	4.76		36,964	1,760	
			5.00	4.88	37,169	1,858	
NML-040408	M	46	6.42		28,498	1,830	
			6.60	6.51	28,205	1,862	
NML-041008-1	F	61	5.73		47,148	2,702	
			5.84	5.79	46,358	2,707	
NML-041008-2	F	33	3.18		31,489	1,001	
			3.54	3.36	32,469	1,149	
NML-042408	F	55	5.14		29,841	1,534	
			6.11	5.63	30,014	1,834	
NML-043008	M	55	6.57		32,903	2,161	
			6.19	6.38	32,152	1,990	
MEAN				5.23	34,011	1,784	
SD				1.13	5,782	508	
CML-050608			9.65		38,196	3,686	
	M	52	9.02	9.34	37,943	3,422	Poorly Controlled, 35.8% FISH +
CML-051308			5.7		34,617	1,973	
	F	57	5.74	5.72	34,547	1,983	Chronic Phase, 24.8% FISH +
CML-061808			5.98		34,752	2,078	
			5.54	5.76	34,993	1,939	Chronic Phase
MEAN				6.94	35,841	2,514	
SD				2.08	1,734	812	

A summary of our analyses of peripheral blood samples from healthy donors (NML) and subjects with CML is depicted in **Table 1**. Samples were run in duplicate with average values and standard deviations (SD) depicted. We initially tested and standardized the cell isolation and staining protocols using samples from healthy individuals, which were not included in the results. Clinical characteristics of the CML subjects are also shown. It should also be noted that an additional sample from an individual with CML was not interpretable because of poor cell viability.

The average percentage of CD4+ lymphocytes that were CD25 and FoxP-3 positive in
Healthy individuals was 5.23% ± 1.13
CML subjects 6.94% ± 2.08
The percentages of CD4+CD25+FoxP-3+ T-lymphocytes from healthy and CML subjects overlap, and numbers from CML subjects are small from a statistical standpoint. However, the initial trend is towards a higher number of regulatory T-cells in the CML population, which would support our initial hypothesis that T_{regs} may be suppressing a host anti-CML response. If one examines the absolute numbers of CD4+CD25+FoxP-3+ T-lymphocytes in the periphery, the difference is accentuated with healthy individuals having **1,784 ± 508 vs 2,514 ± 812** for subjects with CML. Of note is that the individual with CML with poorly controlled disease (CML-050608) had the highest numbers of circulating T_{regs} (9.34%). The full FACS profiles of all subjects can be provided upon request.

We continue to examine subjects with CML using the above protocol(s) to increase our CML sample size for greater statistical significance, and are now starting to look for functional characteristics of the CML T_{reg} populations (Task 2, below). We initially experienced some difficulties in obtaining samples from CML subjects due to scheduling problems (it was difficult to arrange for informed consent for our study before the subjects had scheduled blood samples drawn in the outpatient clinic), or there were simply no subjects with CML available. However, we believe that these problems have been resolved by integration of the timing of informed consent for our study with those of other CML investigation studies.

From our previous work, we have isolated human cytotoxic T-lymphocyte (CTL) clones with BCR-ABL fusion region specificity that are also HLA DRB5*0101 or B*3501 restricted [1]. We are looking for subjects with active CML that whose haplotypes are HLA DRB5*0101 or B*3501 so that primary T_{regs} and CML cells can be isolated from them. If our hypothesis is correct, MHC matched T_{regs} should suppress BCR-ABL peptide mediated CTL responses from our BCR-ABL specific CTL clones. Alternatively, for those individuals with CML with different HLA types, we will evaluate the ability of their T_{regs} to inhibit a mixed lymphocyte response (MLR) which, although perhaps less specific than the inhibition of a BCR-ABL CML specific response, will serve as a measure of their overall T_{reg} suppressor activity.

Task 2. **Immunologic Characterization of CD4+ CD25+ T_{regs} in Individuals with CP CML**

(a) Demonstration and characterization of immune suppression by T_{regs} (Lymphocyte proliferation, gamma IFN ELISPOT, Luminex cytokine secretion assays)
(b) Is suppression specific for CML cells in vitro?
(c) Can suppression be reversed by removing T_{regs} using specific antibodies?

KEY RESEARCH ACCOMPLISHMENTS:

- Identified a trend towards a higher number of CD4+CD25+FoxP-3+ regulatory T-cells in the CML population, supporting our hypothesis that these cells may be regulating host anti-CML responses.

REPORTABLE OUTCOMES:
None

CONCLUSION:
We have identified a trend towards a higher number of CD4+CD25+FoxP-3+ regulatory T-cells in the CML population, supporting our hypothesis that these cells may be regulating host anti-CML responses. We are continuing to accrue information on CD4+CD25+FoxP-3+ regulatory T-cells in subjects with CML, and are now looking at functional correlates of the Treg population in CML.

REFERENCE LIST

1. Sun JY, Senitzer D, Forman SJ, Chatterjee S, Wong KK, Jr. Identification of new MHC-restriction elements for presentation of the p210(BCR-ABL) fusion region to human cytotoxic T lymphocytes. Cancer Immunol.Immunother. 2003;52:761-70.

APPENDICES: None

SUPPORTING DATA: See BODY, above.